In Vivo Demyelination Induced by Intraneural Injection of Anti-Galactocerebroside Serum

A Morphologic Study

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Intraneural injection of rabbit anti-galactocerebroside (anti-GC) serum produced focal demyelinative lesions in rat sciatic nerves. Recipient rats developed a sensory motor deficit of the toes and feet on the side injected with anti-GC serum. Schwann cell abnormalities in recipient nerves were apparent by 20 minutes, followed by myelin splitting and vesiculation over the next 8 hours. Macrophages first appeared in moderate numbers by 15 hours, and degraded myelin was completely phagocytized by 5 days. An acute inflammatory reaction consisting of endoneurial edema, polymorphonuclear cell infiltration, and fibrin extravasation also was prominent. *In vivo* demyelinative activity of rabbit anti-GC serums was removed by pre-incubation with GC or central or peripheral nervous system myelin and was also lost when the serums were heated at 56 C for 30 minutes and injected into nerves of rats previously injected with cobra venom factor. Anti-GC antibodies are present in the serum of rabbits with experimental allergic neuritis (WN-EAN) and encephalomyelitis (WM-EAE) produced, respectively, by immunization with whole peripheral nerve or brain white matter and may play a role in the pathogenesis of demyelination in GC-induced EAN, WN-EAN, or WM-EAE. (Am J Pathol 95:99–116, 1979)

GALACTOCEREBROSIDE (GC), a lipid hapten,[1,2] is a major component of central (CNS) and peripheral nervous system (PNS) myelin.[3] Rabbit anti-GC serum demyelinates organotypic cultures of CNS in the presence of complement [4–6] and inhibits myelination in immature CNS cultures.[5,7] Anti-GC serum also induces demyelination after application to PNS cultures.[6] Since elevated titers of anti-GC antibody are found in serums of rabbits with experimental allergic encephalomyelitis produced by immunization with whole CNS tissue (WM-EAE) [1,6] and with experimental allergic neuritis produced by immunization with whole PNS tissues (WN-EAN),[6] anti-GC antibody could be an important component of the serum demyelinating activity found in these serums *in vitro*.[4–6]

The present work reports the observation that intraneural injection of

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antiserum to GC produces focal demyelinative lesions in *in vivo* peripheral nerve and describes the progression of morphologic changes that occur after injection of anti-GC serum. The sequence of events that proceed after injection of antiserum may have some relevance in dissecting the evolution and pathogenesis of demyelinative lesions in the peripheral nervous system (PNS) of EAN and EAE animals and in patients with Guillain-Barré syndrome (GBS).

Materials and Methods

Preparation of Anti-GC Serums and Control Serums

Five male New Zealand albino rabbits weighing 2.3 to 2.7 kg were used to produce antiserum. Rabbits are known to develop high anti-GC antibody titers.[1,3,5] Each rabbit was injected with 1 ml of inoculum intramuscularly at four sites on the back every 2 weeks for the initial three doses and intraperitoneally 3 weeks after the third injection as a booster. The initial inoculum contained 2 mg of bovine brain cerebrosides (lower spot cerebrosides, 89% with hydroxy fatty acids) (Sigma), 10 mg of bovine serum albumin (BSA), 0.5 ml of complete Freund's adjuvant (CFA) (Difco), and 0.5 ml of phosphate-buffered saline (PBS). The booster inoculum contained 2 mg of cerebroside, 10 mg of BSA, and 1 ml of PBS. Rabbits were bled at each immunization and 3 weeks after booster inoculation. Cerebroside was checked for purity by thin-layer chromatography (TLC) on silica gel G plate developed with chloroform-methanol-water (65 : 25 : 4). Standard lipids were synthetic *N*-lignoceroyl, palmitoyl, and stearoyl dihydrogalactocerebroside (prepared by Sigma following the procedure of D. Shapiro), phosphatidyl ethanolamine, phosphatidyl choline, and sphingomyelin. The major spot (> 99%) had an RF of 0.85 and the minor spot (< 1%) had an RF of 0.88, corresponding, respectively, to cerebrosides with longer (C_{24}) or shorter (C_{18}) fatty acids. Analysis by TLC and gas chromatography following hydrolysis revealed that galactose was the only carbohydrate moiety detectable (> 99.9%)

Control serums were obtained from 5 rabbits before GC inoculation and from 5 control rabbits sensitized and bled following the same schedule used to produce anti-GC serums, except that GC was omitted from the inoculum. All serums were stored in multiple single-use vials at −70 C and were tested within 1 month.

Anti-GC antibody titers were measured by the agglutination of liposomes containing GC and by a radioimmunoprecipitation test employing [H^3]-cholesterol as a marker in the GC-liposomes.[8] Specificity of two anti-GC antiserums with the highest anti-GC antibody titers was tested by comparison of titers when other lipid haptens replaced GC in the liposomes.[8] Only very low titers were obtained by assaying serum antibody with liposomes containing glucocerebroside, lactocerebroside, sphingomyelin, or mixed gangliosides. Antigen-binding capacity of anti-GC serums to CNS myelin basic protein (BP) was tested by coprecipitation of ^{125}I-labeled antigen-antibody complexes.[9] No elevation of antibody titer to CNS-BP was found in anti-GC serums or control serums.

Intraneural Injection of Serums

Male Wistar rats weighing 200 to 250 g were used as recipients because our previous study showed that rabbit anti-GC serum produced identical patterns of *in vivo* PNS demyelination in rats and in rabbits.[10] Fifty microliters of anti-GC serum was injected into the subperineurial portion of one sciatic nerve, and an equal volume of control serum was injected into the contralateral side, using the method described elsewhere.[11]

Evaluation of Antiserum for *In Vivo* PNS Demyelinating Activity

Ten serums were evaluated for *in vivo* peripheral nerve demyelinating activity 2 days after intraneural injection of 50 μl of serum into rat sciatic nerve by our previously described semiquantitative methods.[10] This serum is a mixture of 40 μl of antiserum and 10 μl of guinea pig serum added as a source of complement. The two anti-GC serums with the highest anti-GC antibody activity were tested for heat lability and complement dependency of peripheral nerve demyelinating activity as described previously.[10]

Adsorption of anti-GC antibody was performed by incubating antiserums 9:1 v/v with an emulsion containing 2.9 mg GC, 2.7 mg cholesterol, and 1.3 mg lecithin per 0.29 ml ethanol. The mixture was incubated for 4 hours at room temperature and centrifuged at 20,000*g* for 3 hours at 4 C. Anti-GC serums incubated with similar suspensions either devoid of glycolipid or containing glucocerebroside served as controls. Adsorption of anti-GC serums with bovine CNS or PNS myelin or with bovine liver was performed as described previously.[10]

Sequential Morphologic Study

The anti-GC serum with the most potent demyelinating activity by morphologic criteria was selected to study the chronologic changes of *in vivo* peripheral nerve demyelination. This serum was obtained from a rabbit 3 weeks after booster immunization and had an anti-GC antibody titer of 1:512 by the agglutination assay. This serum was also used for adsorption studies. Two or more rats were examined 20 minutes, 1 hour, 3 hours, 5 hours, 8 hours, 15 hours, 24 hours, 2 days, 3 days, 5 days, and 7 days after intraneural injection. Rats were evaluated clinically at frequent intervals after injection for clinical evidence of sciatic nerve dysfunction. After pentobarbital anesthesia was induced, their nerves were fixed *in situ* with 3.6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. To examine the earliest postinjection times, animals were fixed by perfusion through the abdominal aorta with physiologic saline, followed by glutaraldehyde buffer. Segments of sciatic nerves injected with both anti-GC and control serum were cut into four 1.5-mm portions and processed for light and electron microscopic studies.[11] Portions of the nerves were also processed in 66% glycerin solution after postfixation in OsO_4 for nerve fiber teasing.

Results

Demyelinating Activity of Anti-GC Serums

Seven serums from 5 GC-sensitized rabbits produced focal demyelinative lesions in rat peripheral nerves. These serums had anti-GC antibody titers above 1:64, as tested by the agglutination method.[8] Three other serums with anti-GC antibody titers less than 1:32 did not produce significant *in vivo* peripheral nerve demyelination. Ten control serums had anti-GC antibody titers less than 1:8, and none had measurable demyelinating activity in recipient nerves.

Specificity of Anti-GC Serums

Incubation of the two most potent anti-GC serums with either lyophilized purified bovine CNS myelin or PNS myelin removed the *in vivo* demyelinating activity; the activity of these two serums persisted after

incubation with bovine liver. Incubation of anti-GC serums with the mixture of GC, cholesterol, and lecithin removed demyelinating activity; incubation with similar suspensions devoid of glycolipid or containing glucocerebroside did not. Loss of *in vivo* demyelinating activity after incubation with CNS and PNS myelin, and with GC, always was accompanied by a drop in antibody titer to 1 : 32 or lower. After incubation with liver or lipid suspensions without GC, the anti-GC serums maintained titers above 1 : 64. Demyelinating activity of two anti-GC serums was lost when they were heated at 56 C for 30 minutes and injected into sciatic nerves of rats previously injected with cobra venom factor.

Clinical Observations

A few hours after anti-GC serum injection all rats had ipsilateral toe movement weakness. At 5 hours, complete paralysis of toe movements, weakness of ankle motion, and decreased response to pin prick were noted on the anti-GC serum-injected side. Clinical signs began to improve gradually after the seventh day in most cases. The control serum-injected side remained normal in all animals.

General Morphologic Observations

Macroscopic findings of the anti-GC serum-injected nerves at the time of dissection were usually limited to mild swelling, less than 1 cm long, of the injected segment. Herniation of nerve fibers through the perineurium was not present. Punctate hemorrhages and dilatation of epineurial vessels were occasionally seen. The cross-sectioned surface of anti-GC serum-injected nerves appeared slightly darker than that of controls.

By light microscopy at 24 hours, both anti-GC and control serum-injected nerves shared several changes at the site of subperineurial injection. An area of needle artifact, usually less than 5% of fascicle, consisted of crushed myelin sheaths and a few fibers showing early wallerian degeneration. In the epineurium there was invariably a degree of inflammation, and within the endoneurium there were a few polymorphonuclear cells and degranulated mast cells. A mild degree of edema and endothelial cell swelling of vessels was present.

The microscopic lesion within anti-GC serum-injected nerves was approximately 2 cm in length, extending proximal and distal to the injection site and occupying more than half the fascicular area in cross section. Proximally, the demyelinative effect of serum was present without evidence of needle artifact (Figure 1). Distally, axons undergoing wallerian degeneration were sometimes noted. There was a predilection for lesions remote from the injection site to be located in subperineurial regions.

Sequential Morphologic Observations

Twenty Minutes

Twenty minutes after anti-GC serum injection, the earliest time of pathologic examination, ultrastructural changes of Schwann cells were identified in regions away from the injection site. Some Schwann cells associated with smaller myelinated fibers had mildly dilated endoplasmic reticulum (ER) and swollen mitochondria. Others showed slight opening of their external mesaxons. Occasionally, Schwann cells distant from the injection site showed mushroom-shaped or irregular outfoldings of their plasma membrane. Schwann cell processes surrounding unmyelinated fibers at times retracted from their basement membranes, and axons became bared over part of their circumferences.

One to Five Hours

Between 1 and 5 hours after anti-GC serum injection, the nerves had subtle changes that could be detected by light microscopy. Polymorphonuclear cells increasingly infiltrated the endoneurium. The cytoplasm of Schwann cells failed to stain with toluidine blue and their nuclei were pyknotic. Under the electron microscope Schwann cell cytoplasm was rarefied (Figure 2) or occasionally electron-dense. Myelin sheaths associated with these Schwann cells showed vesicular disruption starting at both the inner and outer myelin lamellae (Figure 2). In longitudinal sections, Schmidt-Lanterman (SL) clefts of nerve fibers were widened and paranodal myelin was retracted. Ultramicroscopically, these cleft widenings consisted of myelin splitting, vacuolation, and vesiculation (Figure 3). The frequency of Schwann cell and myelin changes was greatest around the site of injection and decreased in a graded fashion in areas remote from the injection site. By 5 hours large mononuclear and polymorphonuclear cells had infiltrated into the perineurium, and there was edema and destruction of perineurial lamellae. Despite the adjacent perineurial inflammation, mononuclear cells rarely were found in the endoneurium.

Eight Hours

Extensive myelin vesiculation affected many nerve fibers. This pattern of demyelination could be recognized on light microscopy as a gray halo surrounding individual axons (Figure 4). Phagocytic mononuclear cells were seldom found in the endoneurium and never in association with vesiculated myelin.

Fifteen Hours to Three Days

Many large mononuclear cells with polysome-rich cytoplasm and pseudopodia appeared in the endoneurium at this time. They were present in and around endoneurial blood vessels and were considered to be monocytes. Macrophages also were seen, sometimes associated with demyelinating or normal-appearing myelinated nerves (Figures 5 through 7). The number of demyelinated axons increased during this period. By examining teased single fibers, we demonstrated that demyelination was segmental (Figure 8), usually extending over several internodes. Attachment of debris-laden macrophages was a frequent feature of the demyelinated segments in cross section. Schwann cells associated with nerve fibers which were invaded by macrophages usually had degenerative changes consisting of amorphous granular and membranous profiles and swollen mitochondria. However, Schwann cells in areas distant from the injection site showed only mild dilation of smooth ER and increased numbers of caveolae in their plasma membranes. Phagocytosis of myelin debris by Schwann cells was seldom observed. Unmyelinated fibers were frequently enclosed within a basement membrane but were unassociated with Schwann cell processes. Changes within axoplasm were observed rarely; axonal shrinkage of some demyelinating fibers was indicated by wrinkling and redundancy of axolemmal membranes. Fibrinous exudates and extravasation of red cells were occasionally present around vessels. Subperineurial and endoneurial edema was pronounced.

Five to Seven Days

Most nerve fibers within the lesion were completely demyelinated and were associated with debris-laden macrophages after 5 days. On occasion Schwann cell cytoplasm rich in polysomes and rough ER surrounded demyelinated axons. At 7 days, most demyelinated axons were encircled by Schwann cells (Figure 9).

Control Serum-Injected Nerves

Schwann cells and nerve fibers were generally normal 20 minutes to 7 days after injection of control serum. On occasion we found minimal changes of Schwann cell cytoplasm consisting of mild dilatation of ER and swelling of mitochondria during the first 8 hours after injection of control serum. A few demyelinated fibers were found on careful search of several control serum-injected nerves.

Discussion

The present study has demonstrated that intraneural injection of antiserum to galactocerebroside can produce *in vivo* primary demyelination in a circumscribed area of the recipient peripheral nerve. The demyelinative lesion is characterized by Schwann cell changes and myelin destruction followed by phagocytic mononuclear cell invasion and by a rapidly evolving acute inflammatory response. Demyelinating activity of anti-GC serum, as tested by intraneural injection, is adsorbed by purified CNS and PNS myelin and GC but not by glucocerebroside or liver. Activity is lost after heating serum at 56 C for 30 minutes. The demyelinating activity of anti-GC serum is therefore probably antibody-dependent complement-mediated.

Intraneural injection of serums from rabbits with WN-EAN and WM-EAE also produces primary focal demyelination in recipient rat sciatic nerves.[10-13] The patterns of demyelination and the evolution of demyelinative lesions induced by both WN-EAN and WM-EAE serum are identical to those seen in this study. Schwann cell changes, myelin splitting and vesiculation, macrophage phagocytosis of myelin, and the association of an acute inflammatory reaction are responses common to the three antiserums. WN-EAN and WM-EAE serum with *in vivo* PNS demyelinating activity also have elevated anti-GC antibody titers.[12] Demyelinating activity in these serums, as tested by intraneural injection, is also adsorbed with galactocerebroside but not with glucocerebroside or mixed gangliosides,[12] as were the anti-GC serums in this study. It seems that anti-GC antibody is at least one major antibody responsible for the production of *in vivo* PNS demyelination in both WN-EAN and WM-EAE serum. In contrast, antiserum to CNS-BP does not produce significant myelinotoxicity when injected into peripheral nerve [13] or in myelinated cultures of CNS tissue,[14,15] in spite of the well-established encephalitogenicity of CNS-BP.[16,17]

Demyelinative patterns produced by intraneural injection of anti-GC serum may be compared with those described in myelinated PNS or CNS cultures after application of anti-GC serum, WN-EAN serum, WM-EAE serum, or GBS patient serum and in the PNS of EAN or EAE animals and of GBS patients. Changes produced in organotypic CNS cultures after application of anti-GC serum [4] were described as somewhat different from those resulting from WM-EAE serum.[18] Anti-GC serum reportedly produced demyelinative changes which progressed more slowly and consisted primarily of pronounced intramyelinic swelling [4]; WM-EAE serum produced rapidly evolving change consisting of "smudging" of myelin, large

intramyelinic swellings, oligodendroglial degeneration, and astrocytic phagocytosis of myelin and myelin debris.[18] The differences are inconsistent, for we found that anti-GC serum can rapidly demyelinate organotypic CNS cultures and that the patterns of demyelination also include myelin vesiculation and "smudging" and invasion of phagocytic cell processes.[6] In addition, we found that the pattern and progression of anti-GC serum-induced *in vivo* PNS demyelination is identical to that produced by intraneural injection of WM-EAE serum.

Swelling and rarefaction of Schwann cell cytoplasm, as observed in the early phase after intraneural injection of anti-GC serum, is not described in the PNS of EAN or EAE animals,[19,20] although Schwann cell changes suggestive of insidious damage are found in the PNS of animals with chronic EAE.[21] Schwann cell abnormalities are also present in the PNS of patients with GBS.[22,23] However, in PNS cultures after application of EAN or GBS serum, Schwann cell alterations are well recognized.[24,25]

Vesicular disruption of myelin, usually associated with Schwann cell damage and occurring prior to appearance of macrophages, is one of the conspicuous features of anti-GC serum-induced *in vivo* demyelination. This type of myelin disruption is described in the PNS of animals with EAN,[26] the CNS and PNS of animals with EAE,[19,27,28] and the PNS of patients with GBS.[22,23,29] It also can be seen to some extent in conditions other than demyelinative disorders.[30-32] Application of lysocompounds,[33,34] bee venom [35] (which contains phospholipase A [36]), or calcium ionophore [37] into peripheral nerves can produce extensive myelin vesiculation. Myelin vesiculation produced by intraneural injection of anti-GC serum may share a mechanism with these conditions. Myelin lamellae sensitized with anti-GC antibody could be damaged by activated complement fragments. This may result in rapid autolysis of myelin by release of hydrolytic enzymes such as phospholipase A or other lysocompounds from damaged myelin membrane, perhaps mediated by local elevation of calcium.[37]

Demyelination by macrophage phagocytosis is a late feature after anti-GC serum injection. It appears to be a secondary phenomenon, because several types of myelin destruction precede macrophage-associated demyelination by several hours. In the PNS of animals with EAN and EAE, demyelination is reported to occur by direct attack of (phagocytic) mononuclear cells as a "peeling-off" or "lytic" phenomenon.[20,38] Demyelination by macrophage phagocytosis is also described in the PNS cultures after application of WN-EAN serum [25] and in the PNS of patients with GBS.[22,23,29]

In unmyelinated fibers after intraneural injection of anti-GC serum, we found Schwann cell damage and subsequent disappearance of Schwann

cells. Macrophage invasion into unmyelinated fibers has been described in the PNS of immature rabbits with EAN.[39] In an autopsy study of patients with GBS, involvement of sympathetic ganglia has been noted.[40]

Minor changes common to both control and experimental nerves could result from effects of needle insertion and possibly reactions to heterologous serum and its acute-phase reactants. These problems are discussed in detail elsewhere.[11] Previous studies have shown that a variety of antiserums against non-neural antigens such as liver or BSA do not produce significant demyelination in rat sciatic nerve.[10-13]

An immunologic mechanism is probably responsible for the initial phase of anti-GC serum-induced demyelination of nerve. Myelin contains GC as a major constituent and Schwann cell plasma membrane also may include GC. Anti-GC antibodies could sensitize myelin and possibly Schwann cell membrane, which are then damaged by bound and activated complement fragments. Schwann cell changes also might occur secondary to immunopathologic destruction of myelin sheaths.

The possible importance of anti-GC antibodies in the pathogenesis of EAN is emphasized by our recent studies which demonstrate that rabbits repeatedly immunized with GC developed experimental allergic neuritis (GC-EAN). This disorder is manifested primarily by subacute or chronic onset of ataxia and sensory motor disturbances of limbs.[41] Pathologic examination of rabbits with GC-EAN revealed multiple demyelinative and remyelinative lesions in association with macrophages in roots, dorsal root ganglia, and, less often, the peripheral nerves. The pathologic features differed from those of experimental allergic neuritis produced by sensitization with whole peripheral nerve homogenates,[42] peripheral nerve myelin P_2 basic protein,[43] or its peptide [44] in that perivenular infiltration of small lymphocytes was infrequent. The paucity of perivascular lymphocytic infiltration and the prevalence of lesions in areas where rabbits have an incomplete blood-nerve barrier [45-47] to serum proteins raises the possibility that the demyelinative disease induced by GC in these rabbits is mediated primarily by circulating factors.

Demyelinative lesions induced by anti-GC serum share many pathologic features with peripheral nerve and root changes of animals with EAN, EAE, and GC-induced EAN animals and of patients with GBS. Anti-GC antibodies in GC-EAN serum, WN-EAN serum, or WM-EAE serum may play a role in the demyelinative lesions in these disorders.

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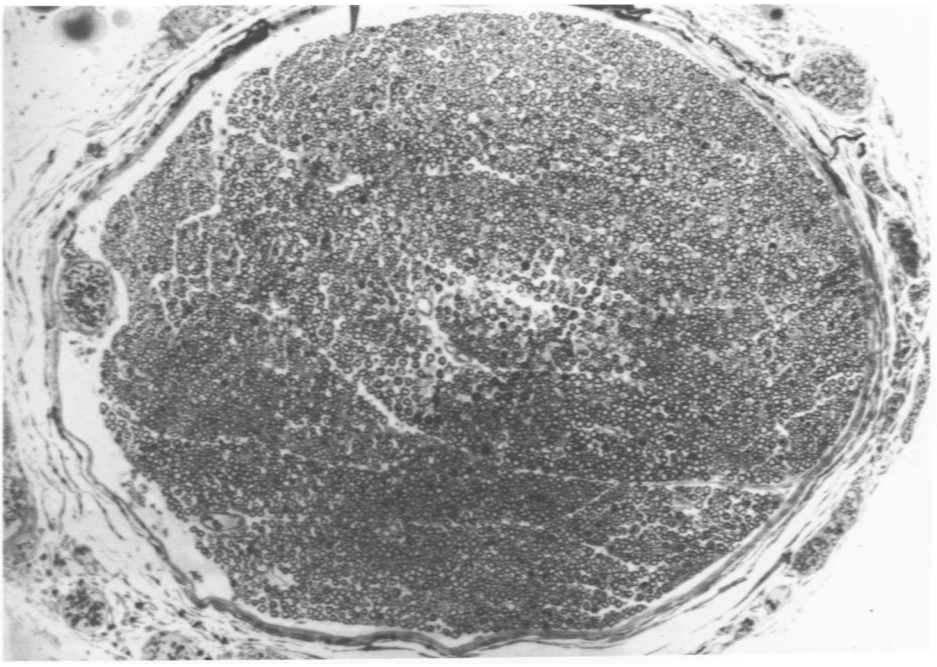


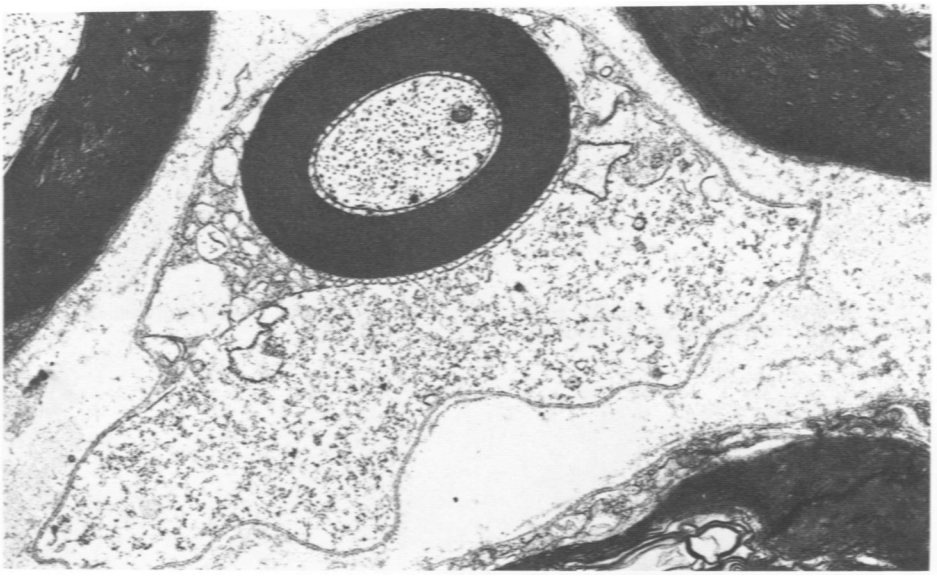
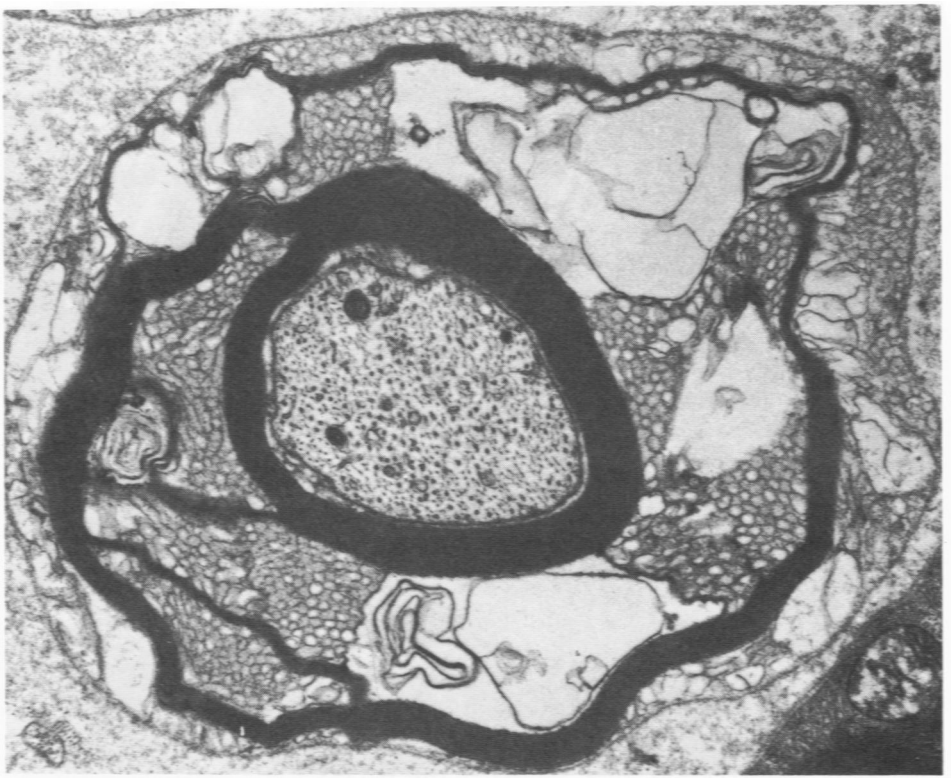


Figure 1—Low magnification cross section of a rat sciatic nerve 2 days after anti-GC serum injection. There is subperineurial and endoneurial edema within the fascicle. (×58) **Figure 2**—One hour after anti-GC serum injection. Schwann cell cytoplasm is rarefied, with amorphous granular material and dilated rough endoplasmic reticulum. Early myelin vesiculation is apparent at both outer and inner myelin layers. (×7900)

Figure 3—Myelinated nerve fiber 5 hours after anti-GC serum injection. There is splitting, vacuolation, and vesiculation of myelin lamellae, probably at a Schmidt-Lanterman cleft. Schwann cell cytoplasm is no longer discernible, but the axons appear normal. (×28,200)

Figure 4—Eight hours after anti-GC serum injection. Many axons are surrounded by a gray halo (*arrow*). Note scattered polymorphonuclear cells. (×350)



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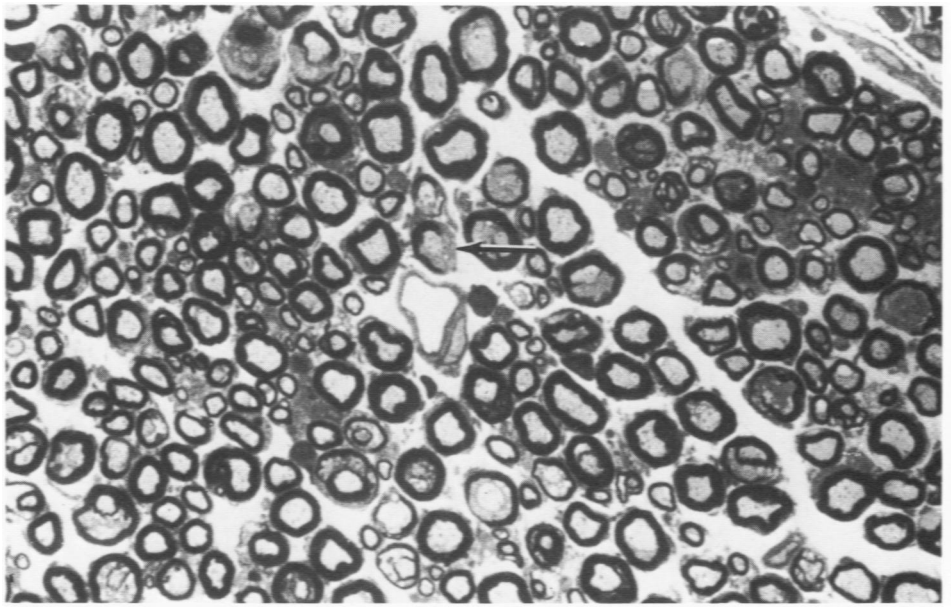

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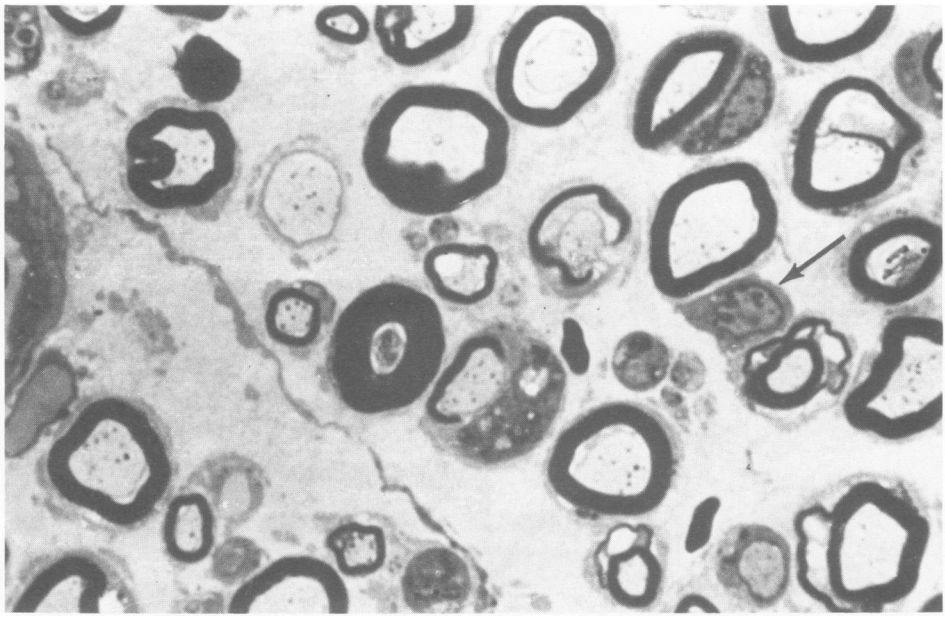

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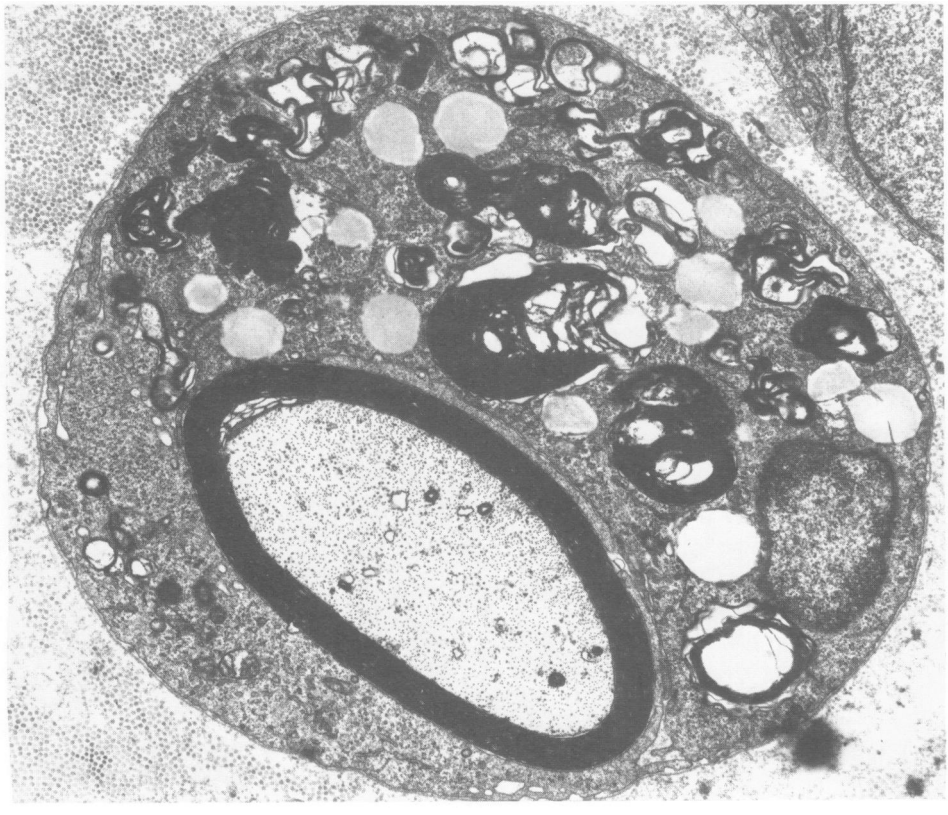
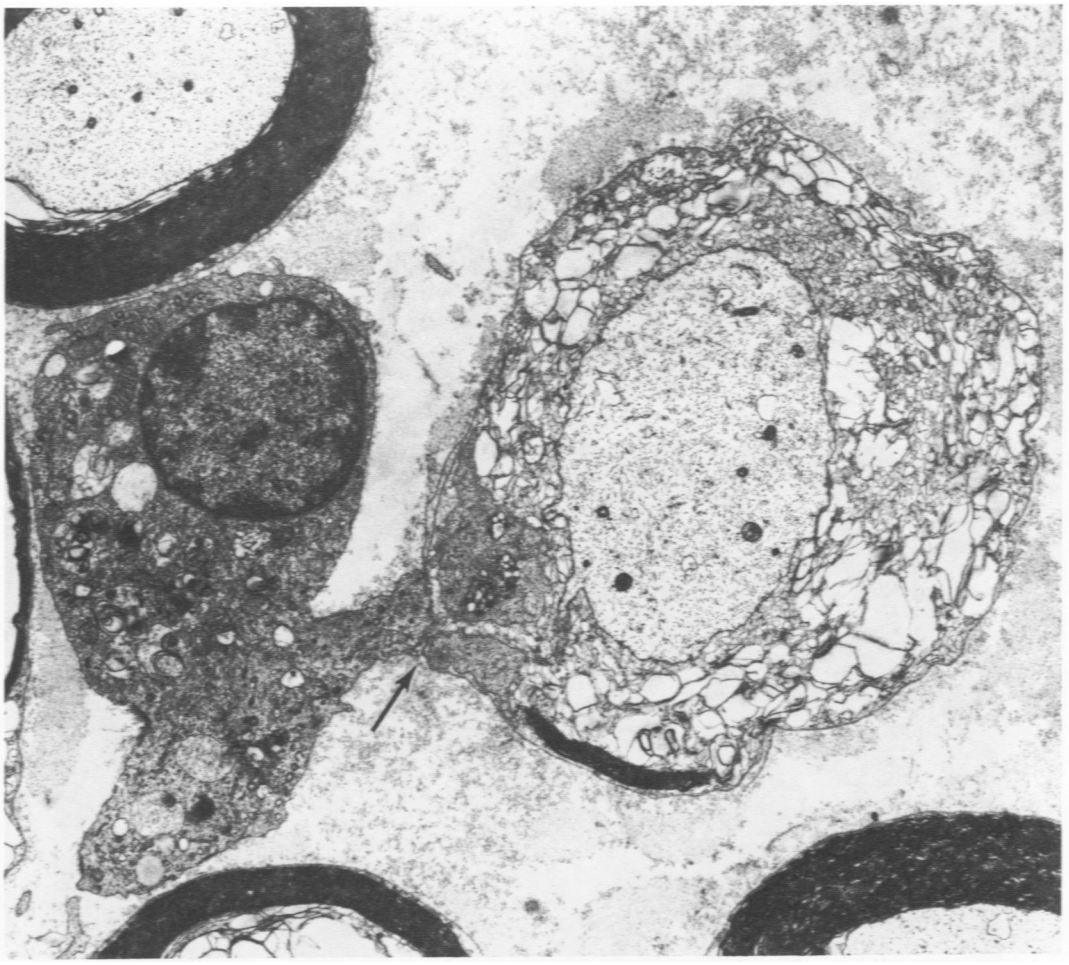


Figure 5—Two days after anti-GC serum injection. Large mononuclear cells and macrophages (*arrow*) have infiltrated the endoneurium and surrounded partially demyelinating or apparently normal fibers. A completely demyelinated fiber is present at the *left center*. (×950) **Figure 6**—Two days after anti-GC serum injection. A normal-appearing myelinated nerve fiber is encircled by debris-laden macrophages that have replaced Schwann cell cytoplasm. (×6900)



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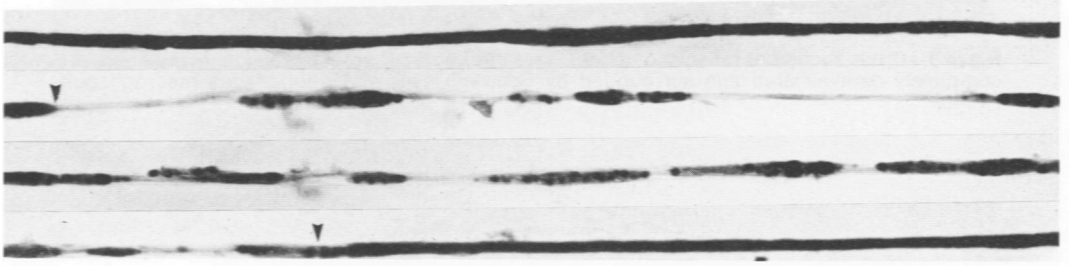

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Figure 7—Two days after anti-GC serum injection. A phagocytic mononuclear cell is anchored (*arrow*) to an extensively vesiculated fiber. The axon appears normal. (×9400) **Figure 8**—Sequential internodes of a single teased myelinated fiber 3 days after anti-GC serum injection. There is segmental demyelination (*between arrowheads*). Patchy myelin debris attached to the demyelinated segment appears to be within macrophages. (×180)

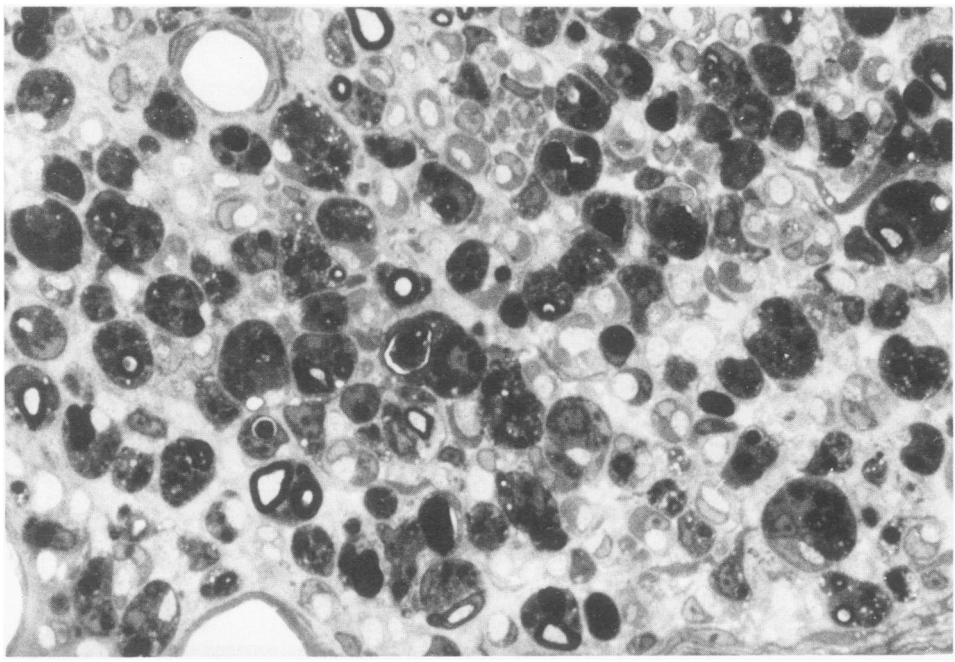

Figure 9—Cross section of rat sciatic nerve 7 days after anti-GC serum injection. Many fibers are completely demyelinated and surrounded by Schwann cells and debris-laden macrophages. (×940)